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SMN GENE DELETIONS ASSESSMENT AND PRENATAL DIAGNOSIS IN FAMILIES OF CHILDREN WITH THE SYNDROME TABRIZ SPINAL ATROPHY – MUSCLE

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ABSTRACT
Muscle atrophy syndrome - Spinal (SMA) is one of the common diseases of muscle - nerve, with progressive paralysis is due to the alpha motor neuron in the spinal cord becomes waste. SMN1 and SMN2 gene expression in SMA by only a single nucleotide in exon 7 are different. Homozygous deletion of exon 7 in the SMN1 gene is the most common mutation observed. Compound heterozygosity small proportion of patients with a point mutation in one allele and the other allele are removed. In other cases the disease does not appear to be the result of a change in SMN1. In spinal atrophy - muscle, SMN2 unable to compensate for the shortage caused by the deletion of exon 7. The aim of the present study was to estimate the prevalence of common deletion of exon 7 in the SMN1 gene families in Tabriz, in order to determine the status of the carrier and prenatal diagnosis. 119 families with a history of child with SMA and determined that the deletion of exon 7 in the child with the parents were carriers and Also well as 42 cases of prenatal diagnosis for couples fetus was a carrier. Of the families surveyed, 127 families with a history of SMA1, 24 families with a history of type II and 11 families with a history of type III disease who have had children. In families with a history of a patient, 89 families had both parents carry the deletion of exon 7 and in 26 families, one of them was carrying. The frequencies in families with a history of type II SMA 16 and 1, respectively, and in families with a history of type III SMA was 2,3 respectively. Investigation of fetal samples showed that 11 of 63 samples (5.17%) were diagnosed. Except for two, all patients were homozygote for deletion of exon 7 (7.96% of patients). The study showed that molecular analysis of the two alleles of exon 7 SMN1 appropriate method for the removal and reliable diagnosis of patients to determine the carrier and prenatal diagnosis in families with patients with SMA is.

KEYWORDS: SMN1, SMN2, SMA, Exon 7, PCR.

INTRODUCTION
SMA Spinal muscular atrophy is a group of genetic diseases that are associated with the analysis, causing muscle weakness and spinal cord motor neurons in most cases leads to death of the infected person[1]. This disease is the most common autosomal recessive disorder after cystic fibrosis The prevalence is considered to have a baby every 10,000 births and carrier frequency of one in four to one in fifty people is different [2]. SMA anterior horn cells of the spinal cord will be determined by analyzing[3]. In this disease, the nerve cellsthat control muscle movements proximal voluntary, mobility destroyed[4]. That may result from intrauterine period or at any time after it is created and garlic have fast or slow[5]. The earlier the disease starts to progress faster[6]. Infants infected at birth or in the first few months of life are poor usually suffer from paralysis and crippled limbs throat area, respiratory failure and Death in the first few years of life are the early and premature death, disease (werding hoffman) is said to be[7]. Mild form of the disease (Kogelberg Volandri syndrome) in late childhood or adolescence with muscle weakness and upper legs started slowly progresses over several decades[8]. SMA types that usually occurs between the ages of course is unpredictable[9].

Proximal spinal muscular atrophy (SMA) is an autosomal recessive disease caused by a genetic defect in the SMN1 gene, which encodes SMN, a protein widely expressed in all eukaryotic cells. SMN is apparently selectively necessary for survival of motor neurons, as diminished abundance of the protein results in loss of function of neuronal cells in the anterior horn of the spinal cord and subsequent system-wide muscle wasting (atrophy).

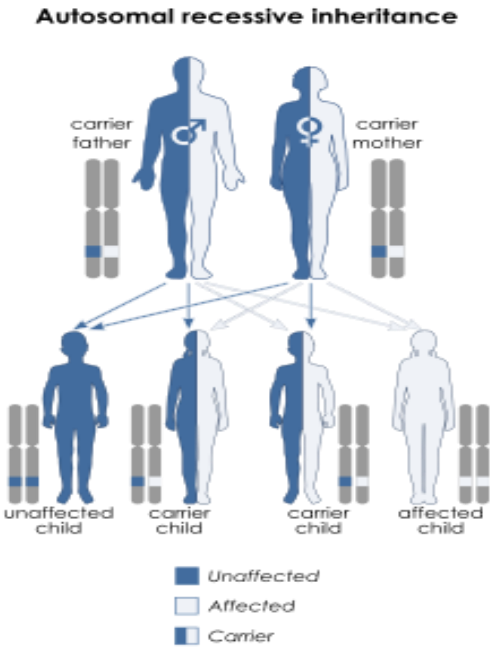
Spinal muscular atrophy manifests in various degrees of severity, which all have in common progressive muscle wasting and mobility impairment. Proximal muscles and lung muscles are affected first. Other body systems may be affected as well, particularly in early-onset forms. SMA is the most common genetic cause of infant death.

The term *spinal muscular atrophy* is used as both a specific term for the genetic disorder caused by deficient SMN, and a general label for a larger number of rare disorders having in common a genetic cause and slow progression of weakness without sensory impairment caused by disease of motor neurons in the spinal cord and brainstem – see spinal muscular atrophies for a comparison chart.

Signs and symptoms

The symptoms vary greatly depending on the SMA type involved, the stage of the disease, and individual factors; they commonly include:

- Areflexia, particularly in extremities
- Overall muscle weakness, poor muscle tone, limpness or a tendency to flop
- Difficulty achieving developmental milestones, difficulty sitting/standing/walking
- In infants: adopting of a frog-leg position when sitting (hips abducted and knees flexed)
- Loss of strength of the respiratory muscles: weak cough, weak cry (infants), accumulation of secretions in the lungs or throat, respiratory distress
- Bell-shaped torso (caused by using only abdominal muscles for respiration)
- Clenched fists with sweaty hands
- Head often tilted to one side, even when lying down
- Fasciculations (twitching) of the tongue
- Difficulty sucking or swallowing, poor feeding
- Weight loss



Spinal muscular atrophy has an autosomal recessive pattern of inheritance.

Spinal muscular atrophy is linked to a genetic mutation in the SMN1 gene.[1]

Human chromosome 5 contains two nearly identical genes at location 5q13: a telomeric copy **SMN1** and a centromeric copy **SMN2**. In healthy individuals, the SMN1 gene codes the survival of motor neuron protein (SMN) which, as its name says, plays a crucial role in survival of motor neurons. The SMN2 gene, on the other hand - due to a variation in a single nucleotide (840.C→T) - undergoes alternative splicing at the junction of intron 6 to exon 8, with only 10-20% of SMN2 transcripts coding a fully functional survival of motor neuron protein (SMN-fl) and 80-90% of transcripts resulting in a truncated protein compound (SMNΔ7) which is rapidly degraded in the cell.[*citation needed*]

In individuals affected by SMA, the SMN1 gene is mutated in such a way that it is unable to correctly code the SMN protein - due to either a deletion occurring at exon 7 or to other point mutations (frequently resulting in the functional conversion of the SMN1 sequence into SMN2). All patients, however, retain at least one copy of the SMN2 gene (with most having 2-4 of them) which still codes small amounts of SMN protein - around 10-20% of the normal level - allowing some neurons to survive. In the long run, however, reduced availability of the SMN protein results in gradual death of motor neuron cells in the anterior horn of spinal cord and the brain. Muscles that depend on these motor neurons for neural input now have decreased innervation (also called denervation), and therefore have decreased input from the central nervous system (CNS). Denervated skeletal muscle is more difficult for the body to control. Decreased impulse transmission through the motor neurons leads to decreased contractile activity of the denervated muscle. Consequently, denervated muscles undergo progressive atrophy.[*citation needed*]

Muscles of lower extremities are usually affected first, followed by muscles of upper extremities, spine and neck and, in more severe cases, pulmonary and mastication muscles. Proximal muscles are always affected earlier and to a greater degree than distal.[*citation needed*]

The severity of SMA symptoms is broadly related to how well the remaining SMN2 genes can make up for the loss of SMN1. This is partly related to the number of SMN2 gene copies present on the chromosome. Whilst healthy individuals carry two SMN2 gene copies, patients with SMA can have anything between 1 and 4 (or more) of them, with the greater the number of SMN2 copies, the milder the disease severity. Thus, most SMA type I babies have one or two SMN2 copies; SMA II and III patients usually have at least three SMN2 copies; and SMA IV patients normally have at least four of them.

However, the correlation between symptom severity and SMN2 copy number is not absolute, and there seem to exist other factors affecting the disease phenotype.[2]

Spinal muscular atrophy is inherited in an autosomal recessive pattern, which means that the defective gene is located on an autosome. Two copies of the defective gene - one from each parent - are required to inherit the disorder: the parents may be carriers and not personally affected. SMA seems to appear *de novo* (i.e., without any hereditary causes) in around 2-4% of cases.

Spinal muscular atrophy affects individuals of all ethnic groups, unlike other well known autosomal recessive disorders, such as sickle cell disease and cystic fibrosis, which have significant differences in occurrence rate among ethnic groups. The overall incidence of SMA, of all types and across all ethnic groups, is in the range of 1 per 10,000 individuals; the gene frequency is around 1:100, therefore, approximately one in 50 persons are carriers.[3][4] There are no known health consequences of being a carrier. A person may learn carrier status only if one's child is affected by SMA or by having the SMN1 gene sequenced.

Finally, there are reports of occurrence of both SMA type I and SMA type II among siblings. Scientific explanation of this phenomenon (intrafamilial variability) has been advanced by Enrico Parano, an Italian researcher of the CNR (The National Research Council of Italy). He suggests that these cases might be due to additional de novo deletion of the SMN gene, not involving the NAIP gene (94).[*citation needed*]

Diagnosis

Prenatal screening is controversial, because of its cost and because of the severity of the disease. Some researchers have concluded that population screening for SMA is not cost-effective, at a cost of $5 million per case averted in USA.[5] Others conclude that SMA meets the criteria for screening programs and relevant testing should be offered to all couples.[6]

Very severe SMA (type 0/I) can be sometimes evident before birth - reduction in fetal movement in the final months of pregnancy. Otherwise it manifests within the first few weeks or months of life when abnormally low muscle tone is observed in the infant (the "floppy baby syndrome").

For all SMA types,[*citation needed*]

- Patient will present hypotonia associated with absent reflexes;
- Electromyogram will show fibrillation and muscle denervation;[7]
- Serum creatine kinase may be normal or increased;[*citation needed*]
- Genetic testing will show bi-allelic deletion of exon 7 of the SMN1 gene – this is conclusive of the disease.

Types

SMA manifests over a wide range of severity, affecting infants through adults. The disease spectrum is variously divided into 3–5 types, in accordance either with the age of onset of symptoms or with the highest attained milestone of motor development.

The most severe form of SMA type I is sometimes termed *SMA type 0* (or *severe infantile SMA*) and is diagnosed in babies that are born so weak that they can survive only a few weeks even with intensive respiratory support. SMA type 0 should not be confused with SMARD1 which may have very similar symptoms and course but has a different genetic cause than SMA.

Development milestone attainment is commonly measured using a specially modified Hammersmith Functional Motor Scale.[8][9][10][11]

The eponymous label *Werdnig-Hoffmann disease* (often misspelled with a single "n") refers to the earliest clinical descriptions of childhood SMA by Johann Hoffmann and Guido Werdnig. The eponymous term *Kugelberg-Welander disease* after Erik Klas Hendrik Kugelberg (1913-1983) and Lisa Welander (1909-2001), who distinguished SMA from muscular dystrophy.[12] Rarely used *Dubowitz disease* (not to be confused with Dubowitz syndrome) is named after Victor Dubowitz, an English neurologist who authored several studies on the intermediate SMA phenotype.[*citation needed*]

Treatment

There is no known cure for spinal muscular atrophy.

Palliative care

Care is symptomatic. Main areas of concern are as follows:

- Orthopaedics — Weak spine muscles may lead to development of kyphosis, scoliosis and other orthopaedic problems. Spine fusion is sometimes performed in SMA I/II patients once they reach the age of 8-10 to relieve the pressure of a deformed spine on the lungs. Patients with SMA might also benefit greatly from various forms of physiotherapy and occupational therapy.
- Orthotics / Splints — Orthotic devices can be used to support the body and to aid walking. For example, orthotics such as AFO's (ankle foot orthosis) are used to stabilise the foot and to aid gait, TLSO's (thoracic lumbar sacral orthosis) are used to stabilise the torso.
- Respiratory care — Respiratory system requires utmost attention in SMA as once weakened it never fully recovers. Weakened pulmonary muscles in SMA type I/II patients can make breathing more difficult and pose a risk of hypoxiation, especially in sleep when muscles are more relaxed. Impaired cough reflex poses a constant risk of respiratory infection and pneumonia. Non-invasive ventilation (BiPAP) is frequently used and tracheostomy may

be sometimes performed in more severe cases;[13] both methods of ventilation prolong survival in a comparable degree, although tracheostomy prevents speech development.[14]

- Nutritional care — Difficulties in jaw opening, chewing and swallowing food might put patients with SMA at risk of malnutrition. A feeding tube can be necessary in SMA type I and more severe type II patients.[15][16][17] Additionally, metabolic abnormalities resulting from SMA impair β-oxidation of fatty acids in muscles and can lead to organic acidemia and consequent muscle damage, especially when fasting.[18][19] It is suggested that patients with SMA, especially those with more severe forms of the disease, reduce intake of fat and avoid prolonged fasting (i.e., eat more frequently than healthy people).[20]
- Mobility — Assistive technologies may help in managing movement and daily activity and greatly increase the quality of life.
- Cardiology — Although heart is not a matter of routine concern, a link between SMA and certain heart conditions has been suggested.[21][22][23][24]
- Mental health — SMA children do not differ from the general population in their behaviour; their cognitive development can be slightly faster, and certain aspects of their intelligence are above the average.[25][26][27] Despite their disability, SMA-affected people report high degree of satisfaction from life.[28]

  Palliative care in SMA has been standardised in the *Consensus Statement for Standard of Care in Spinal Muscular Atrophy* which has been recommended for standard adoption worldwide.

Prognosis

Generally, patients tend to deteriorate over time, but prognosis varies with the SMA type and disease progress which shows a great degree of individual variability.

The majority of children diagnosed with SMA type 0/I do not reach the age of 4, recurrent respiratory problems being the primary cause of death.[29] With proper care, milder SMA type I cases (which account for approx. 10% of all SMA I cases) live into adulthood.[30]

In SMA type II, the course of the disease is stable or slowly progressing and life expectancy is reduced compared to the healthy population. Death before the age of 20 is frequent, although many patients live to become parents and grandparents.SMA type III has near-normal life expectancy if standards of care are followed. Adult-onset SMA usually means only mobility impairment and does not affect life expectancy.

Research directions

Since the underlying genetic mechanism of SMA was described in 1990, several therapeutic approaches have been proposed and investigated. Since a vast number of *in vitro* and animal modelling studies suggest that restoration of SMN levels reverts SMA symptoms, the majority of emerging therapies focus on increasing the availability of SMN protein to motor neurons.[*citation needed*]

The main therapeutic pathways under research as of December 2011 include:[31][32][33][34][35][36][37][38][39]

Gene replacement

Gene therapy aims at correcting the SMN1 gene function through inserting specially crafted nucleotide sequences with the help of a viral vector.[40] In the context of SMA, it is currently being researched using the scAAV9 viral vector at the Ohio State University and Nationwide Children's Hospital, USA, and the University of Sheffield, United Kingdom, as well as by Genzyme Corporation, USA, and Généthon, France. Safety and pharmacokinetics of scAAV9 viral vector has been tested in non-human primates.[41] An AAV9 viral vector is in clinical trials for Hemophilia B.

- scAAV9.CB.SMN – a proprietary biologic under development by AveXis, Inc, USA is in Phase I clinical trial as of April 2014 and has both Fast Track Designation and Orphan Drug Designation in the USA. In preclinical studies, this treatment has resulted in the greatest survival increase achieved to-date in a SMNΔ7 mouse model (median survival of 400 days in treated mice as opposed to 15 days in untreated mice).[42]

***SMN2* gene conversion**

This approach, also known as '*SMN2' alternative splicing modulation*, essentially aims at converting the "backup" *SMN2* gene (which normally produces only a fraction of needed SMN protein) into a fully functional *SMN1* gene so as it is able to code for high quantities of full-length SMN protein. As of 2014, the following compounds are part of clinical programmes:

- Antisense oligonucleotides:[43][44][45]
  - *ISIS-SMNRx* — a proprietary molecule under development by Isis Pharmaceuticals, USA, and as of July 2014, in phase II clinical trials; has *Fast Track Designation* in the USA and *Orphan Medicinal Product Recommendation* in the European Union. According to its manufacturer, it has had remarkable results in earlier human trials and is expected to be available to patients around 2017-2018.
  - *PTK-SMA1* — a proprietary small molecule splicing modulator of the tetracyclines group under development by Paratek Pharmaceutical, USA
- Quinazolines:[46]
  - *RG3039* (formerly, *Quinazoline495*) — a proprietary quinazoline derivative developed by Repligen, USA, and licensed to Pfizer.[47] It has an *Orphan Drug Designation* and *Fast Track Designation* in the USA and *Orphan Medicinal Product Recommendation* in the European Union. As of July 2014, it is scheduled for phase II clinical trials.
- Morpholino oligomers, whose way of action resembles that of antisense oligonucleotides. This

class of compounds is in pre-clinical studies in UK and Australia.

Additionally, the following splicing regulators have been investigated in SMA with some interesting results:

- Sodium orthovanadate — shown to modulate alternative splicing in one study *in vitro*[48]
- Aclarubicin — shown effective in human cells from patients with type I SMA,[49] not trialled any further due to toxicity at required dosage

***SMN2* gene activation**

SMN2 activation aims at increasing expression of the SMN2 gene and thus increasing the amount of full-length SMN available; investigated compounds included:

- Growth hormone
- Histone deacetylase inhibitors:[50]
  - Aliphatic compounds:
    - Butyrates: sodium butyrate and sodium phenylbutyrate — promising *in vitro* and demonstrated effective in mouse models,[51][52][53] proved ineffective in symptomatic patients (probably due to extremely short half-life),[54] still being trialled in pre-symptomatic type I/II infants[55]
    - Valproic acid — formerly used widely on experimental basis due to earlier research showing its effectiveness *in vitro*[56] and in mouse models,[57] in achievable concentrations demonstrated ineffective in patients with SMA[58][59][60] and even shown to aggravate SMA symptoms.[61]
  - Benzamides:
    - M344 — shown very effective in mouse models,[62] so far not trialled in patients with SMA
  - Hydroxamic acids:
    - CBHA, SBHA — shown very promising *in vitro*
    - Entinostat (MS-275) — shown very promising *in vitro*
    - Panobinostat (LBH-589) — shown very effective in mouse models,[63] not trialled in patients with SMA due to toxicity at required dosage
    - Trichostatin A — shown effective in mouse models,[64][65] so far not trialled in patients with SMA
    - Vorinostat (SAHA) — shown effective in mouse models,[66] so far not trialled in patients with SMA
- Hydroxycarbamide (hydroxyurea) — shown effective in mouse models[67] and subsequently commercially researched by Novo Nordisk, Denmark, but demonstrated no effect on patients with SMA in subsequent clinical trials[68]
- Natural polyphenol compounds: resveratrol, curcumin — moderate effectiveness on muscle strength supported by anecdotal evidence from patients and limited research *in vitro*[69][70]
- Prolactin — recently shown effective in mouse models,[71] so far not trialled in patients with SMA
- Salbutamol (albuterol) — demonstrated moderately effective *in vitro*[72] and in three clinical trials involving SMA II/III patients[73][74][75]

SMN stabilisation

SMN stabilisation aims at stabilising the SMNΔ7 protein (the short-lived defective protein coded by the *SMN2* gene) so that it is able to sustain neuronal cells;[76] investigated compounds include:

- Aminoglycosides — shown to increase SMN protein availability[77][78]
  - *TC-007* — a proprietary aminoglycoside antibiotic under commercial development by Tikvah Therapeutics, USA
- Indoprofen[79]

Neuroprotection

Neuroprotection aims at prolonging survival of motor neurons even with low levels of SMN; investigated compounds include:

- β-lactam antibiotics (e.g., ceftriaxone) — shown promising *in vitro*[80][81]
- Follistatin — shown promising *in vitro*[82]
- Olesoxime — a proprietary compound developed by a French company Trophos, currently (2011-2013) under a phase II clinical trial in USA and Europe
- Riluzole — a compound approved for treatment of ALS, currently being investigated for SMA at University of Angers, France
- Thyrotropin-releasing hormone (TRH) — shown promising *in vitro* and in open-label uncontrolled clinical trials[83][84][85] yet did not prove effective in double-blind placebo-controlled trials[35]

Stem cells

Stem cell therapy aims at replacing the damaged motor neurons using corrected stem cells which are implanted in the spinal cord of the patient and are expected to branch out to form regular motor neurons.

To date, there has been no significant breakthrough in the technique since no methods have been found to incite branching of the newly formed axons from the spine up to the peripheral muscles in legs or hands. An experimental programme in SMA was under development by California Stem Cell, USA, but has been suspended before entering human clinical trials.There is no scientific evidence that stem cell injections, sometimes carried out in "stem cell clinics" in a number of developing countries, would offer any benefit in spinal muscular atrophy.[*citation needed*]

Other directions

An unclear mechanism of action is found in the following compounds currently under research:

- *PTC-X* — three proprietary compounds under joint development by PTC Therapeutics, USA, and Hoffmann-La Roche, Switzerland[86]
- *RE-003* — a compound being developed by Retrophin.[87]

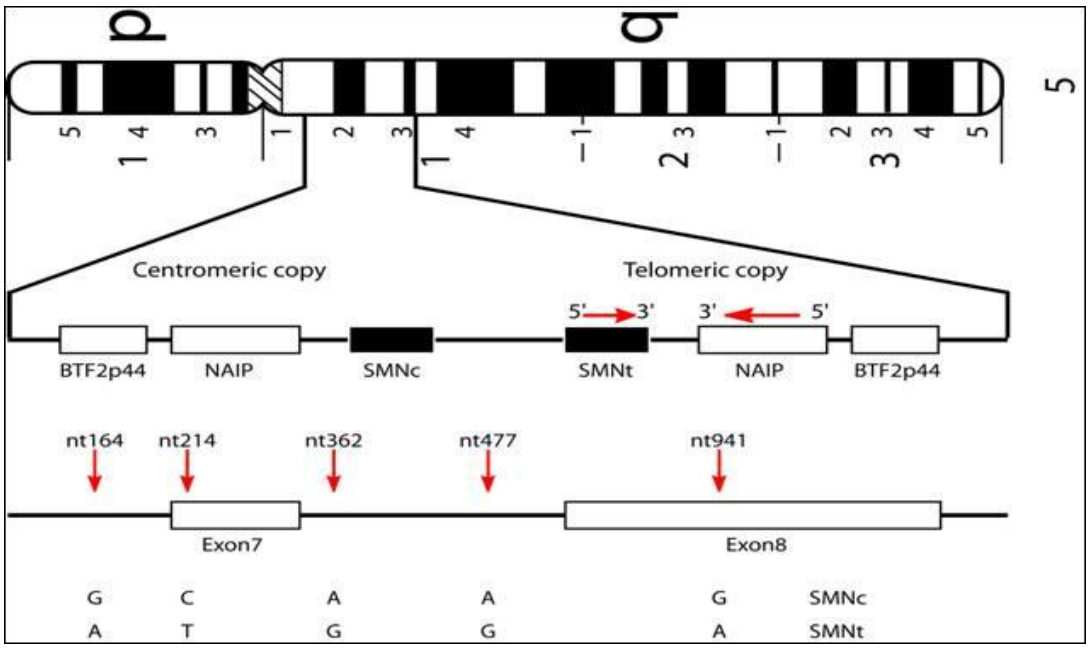
In vivo research is usually conducted using genetically engineered *Caenorhabditis elegans*,[88][89] *Drosophila*,[88][90][91] zebrafish[92] and mouse[93] models;

larger animal models are under development.[94] Patients with SMA can have a chance of participating in research by entering their details into international SMA patient registries. A list of clinical trials targeting SMA can be found here[1].

It has to be noted, though, that SMA therapeutics seem to be most effective when given immediately after birth, then losing their efficacy with the patient's age. This might be related to the variation in time of the needs for SMN protein by neuronal cells. However, this also poses a major therapeutic problem as hardly ever is SMA diagnosed at birth.[95][96]

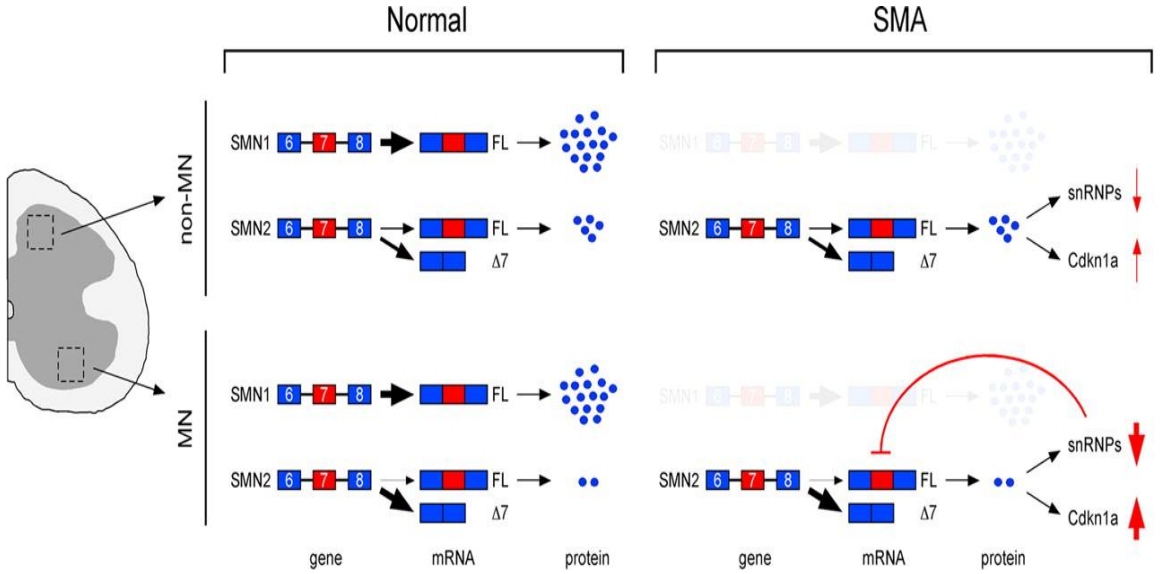
MATERIALS AND METHODS

In this study, families who have children with spinal atrophy - muscle was the second child for prenatal diagnosis with atrophy were examined possible. Families were selected based on SMA consortium. Clinical diagnosis of patients with signs of the disease and in some of them was determined by EMG.



Schematic 1: view of the smn gene exons.

After obtaining consent from families, initially genomic DNA from peripheral blood of patients and their parents with the extracted salt was obtained. Then exon 7 SMN gene patients, their parents Mismatch primer by Mc Andero and colleagues (QPCR) was amplified. In order to detect copies centromeric and telomeric from each other, with an enzyme Dra I to 15 microliters of PCR products amplified from exon 7 was added to the PCR method with appropriate buffer volume was 30 ml. In this way, each tube contains 6.3 units enzyme Dra I was.



Schematics 2: of the normal gene exons of the gene SMA.

All tubes containing the enzyme in a warm bath with a temperature of 37 ° C for 5 hours was made. Products derived from polyacrylamide gel 8% was passed on 10 × TBE buffer and were stained with silver. After digestion with enzymes, in healthy carriers of SMA tapes 188,149,39 pairs of nucleotides.

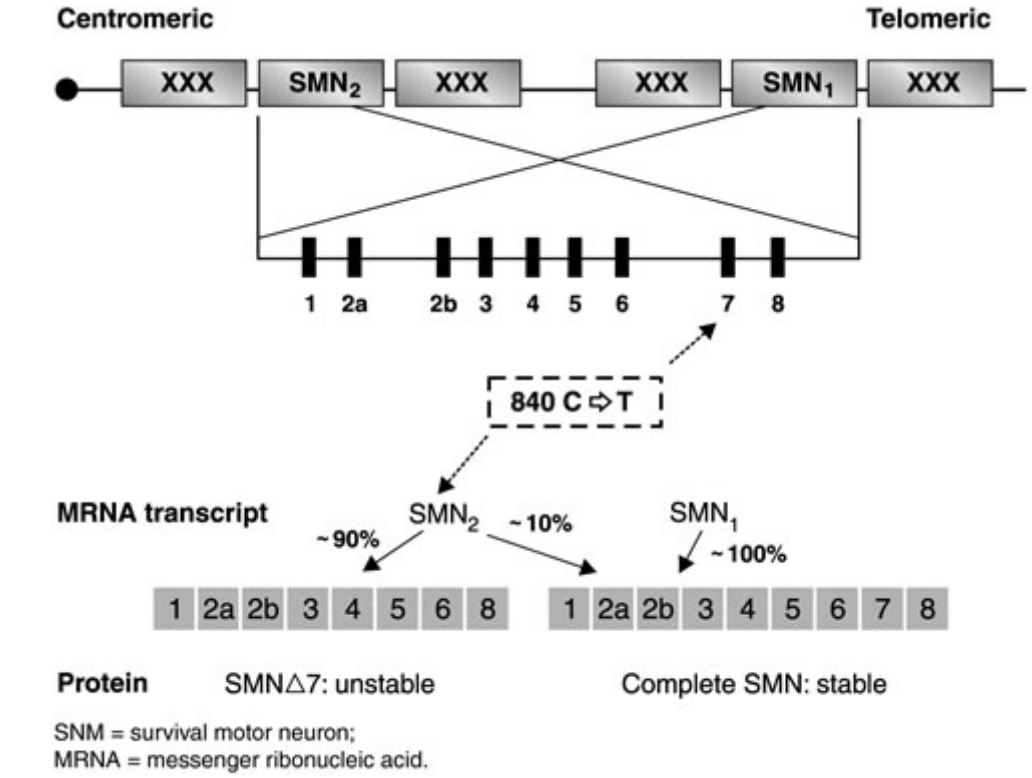
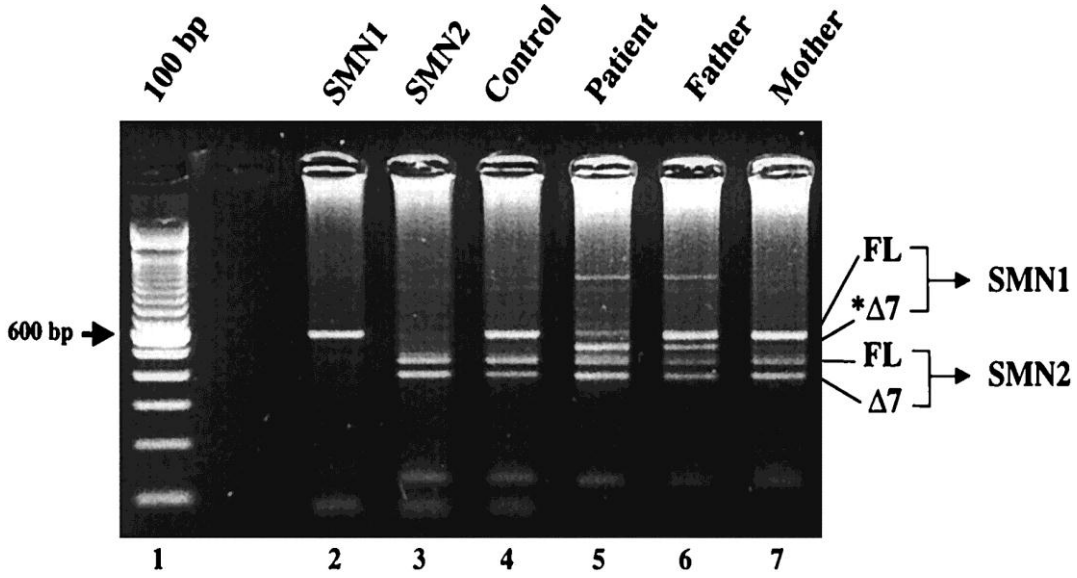
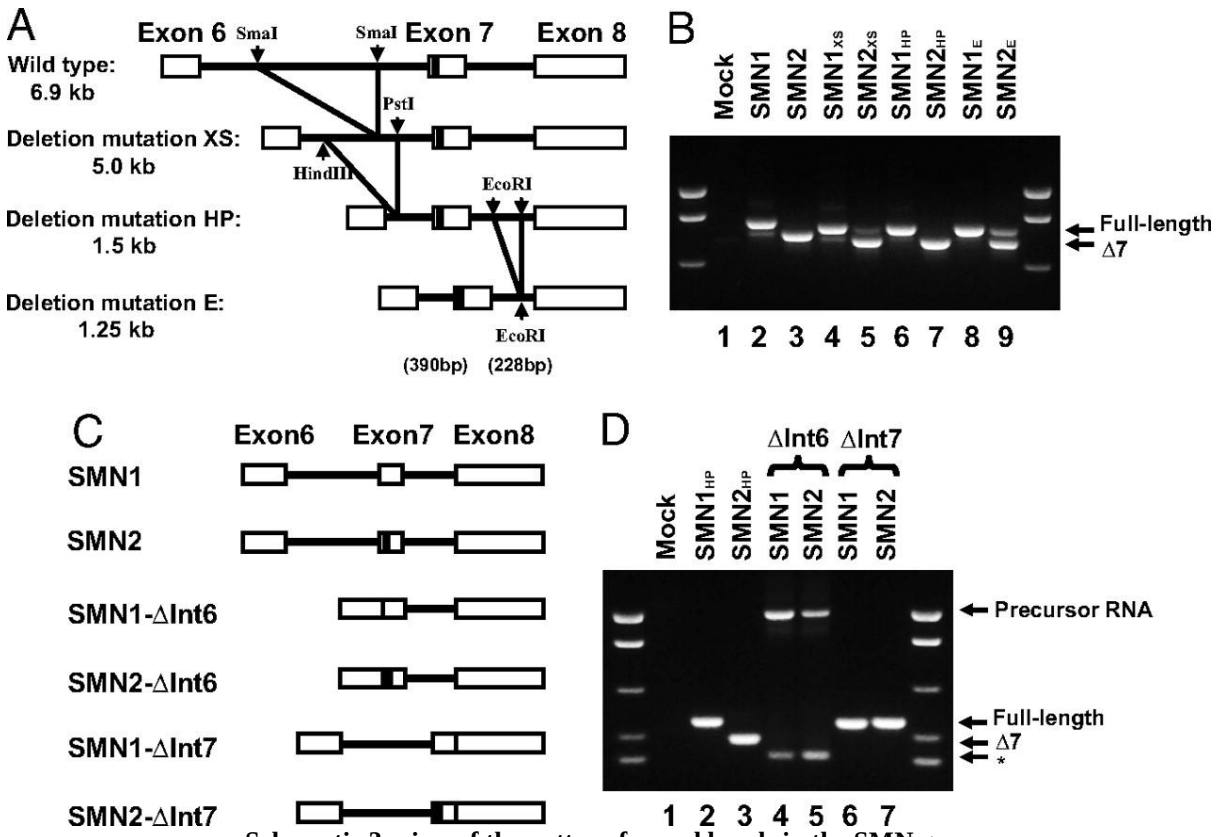


Figure 1 - Structure of the SMN gene in chromosome 5

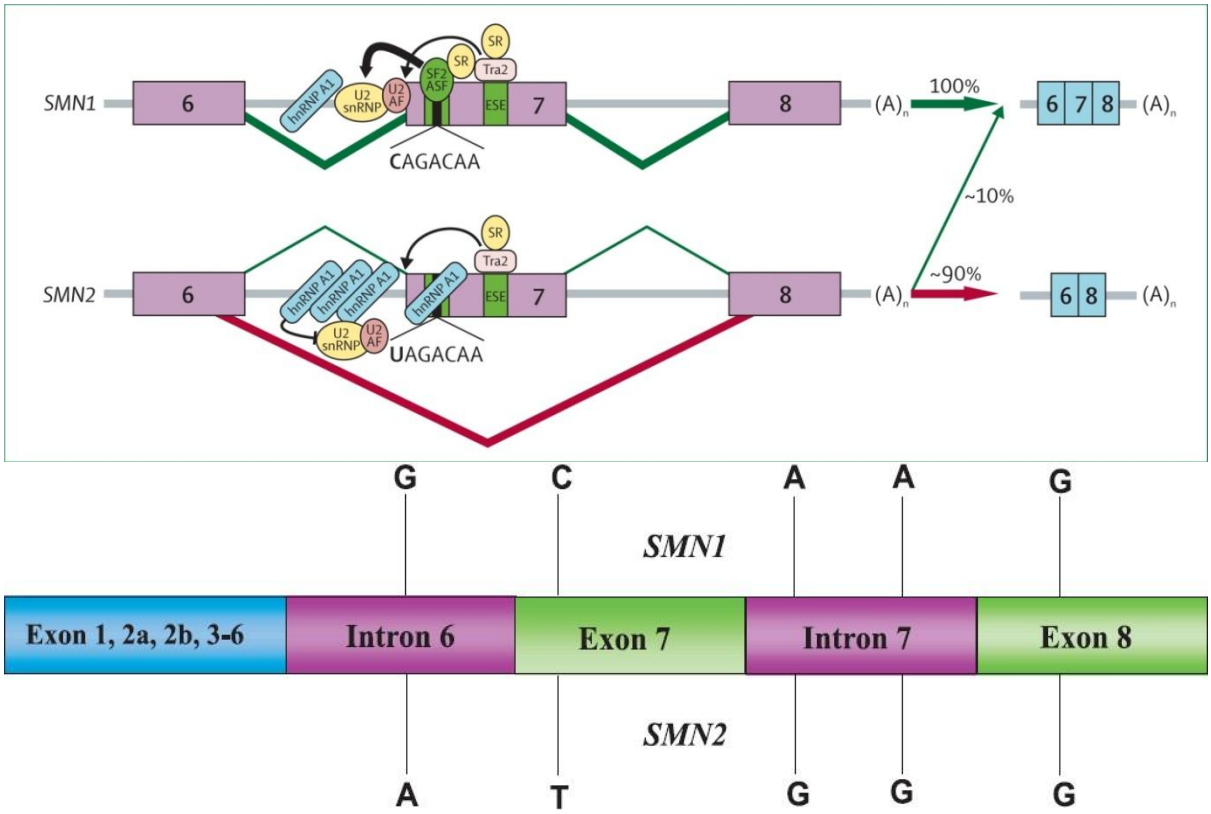
Since the bar 39 bp on the gel can not be seen, only the 188 and 149 bp bands are visible.

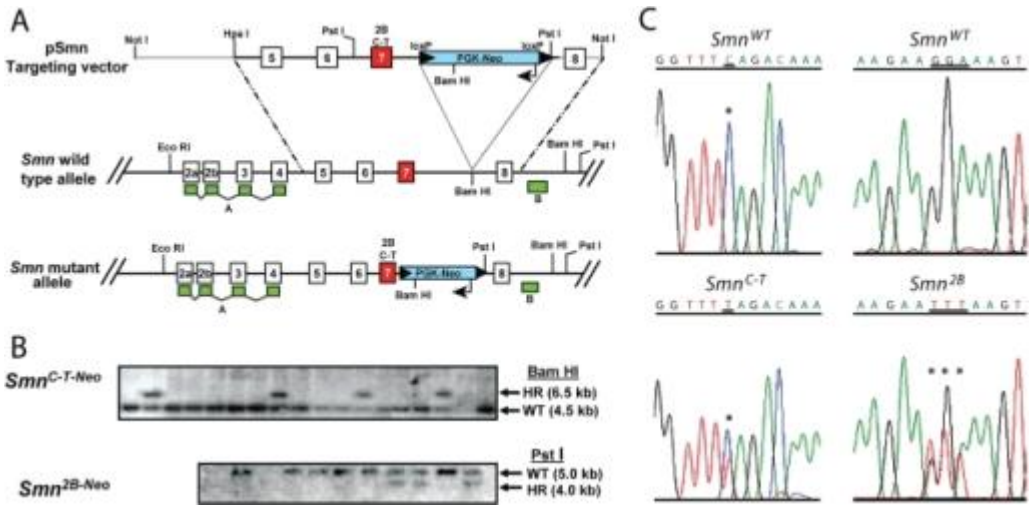




Schematic 3: view of the pattern formed bands in the SMN gene

In patients with SMA after complete digestion with enzymes PCR products 149 bp Dra I only strip is determined. Undigested parts of the SMN gene telomeric and centromeric SMN gene digested parts belong. Patients with SMA are only centromere gene.





Schematic 4: view mutation in SMN gene Along with the nucleotide sequence.

Findings

Using the information obtained, prenatal diagnosis for families with at least one child with SMA were carried out. Chorionic villus samples (CVS) and amniotic fluid (AF) was used. CVS DNA in 29 samples and 11 samples AF with the extracted salt was obtained with the boiling method. Embryonic samples with mutations in exon 7 in terms of the SMN gene were analyzed, the result was positive.

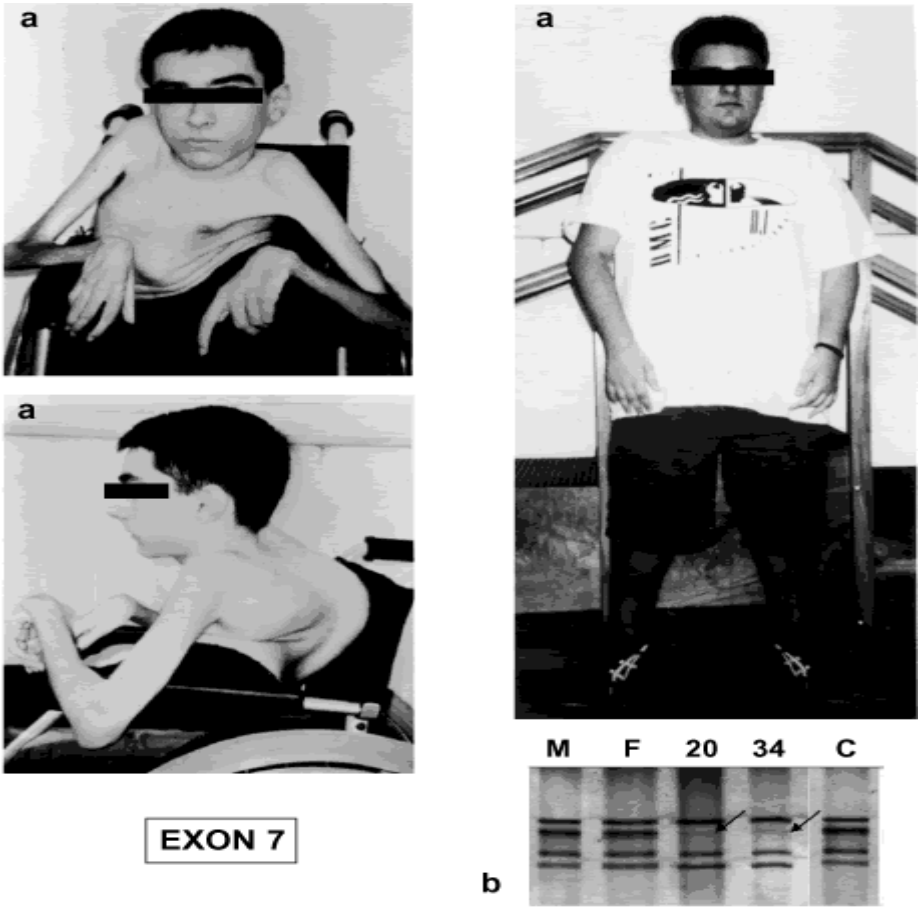


Figure 1 - a, Brothers affected with SMA types II and III. b, Molecular analysis of exon 7 of SMN gene. M - Mother, F - father, cases 20 and 34, and C - control.

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